Selective proteasome inhibitors: modulators of antigen presentation?

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The proteasome is the main nonlysosomal endoprotease in the cytoplasm and nucleus of all eukaryotic cells. It is responsible for the generation of most antigenic peptides as ligands for major histocompatibility complex (MHC) class I proteins. The proteasome hence qualifies as a target for modifying or silencing antigen processing and presentation to cytotoxic T cells, which are important players in transplant rejection and autoimmune disease. The authors summarize recent progress in the understanding of antigen processing by the proteasome and discuss the potential of novel and selective proteasome inhibitors as drugs for suppressing or modifying the cytotoxic immune response.

The search for drugs that can specifically suppress the cytotoxic T-cell response has been intensive for many years[1]. Cytotoxic T lymphocytes (CTLs) are responsible, at least in part, for the aetiology of autoimmune diseases such as insulin-dependent diabetes mellitus and multiple sclerosis[2]. CTLs mediate the rejection of transplanted organs as well as the destruction of virally infected tissues, leading to meningitis and other immunopathological diseases. Currently available immunosuppressive drugs are either corticosteroids or general antiproliferative substances with limited specificity and marked side effects[1]. Even cyclosporin, which interferes with the signal transduction of T cells and has markedly improved immunosuppression in organ transplantation, is poorly tolerated by some patients because of nephrotoxicity[2] and is ineffective in several autoimmune diseases. Suppression of the cytotoxic immune response could be achieved either by preventing the activation and function of CTLs or by interfering with the presentation of antigens through cells of the body. This review concentrates on inhibitors of the proteasome as candidate drugs for selective immunosuppression and for modulating antigen processing in the major histocompatibility complex (MHC) class I restricted immune response.

MHC class I assembly pathway

The antigen receptor of CTLs has a dual specificity for a MHC class I molecule and for a peptide ligand bound in a specialized groove of the MHC class I protein. For binding, the peptide must have a length of 8–11 amino acids and must contain so-called 'anchor residues' at the C-terminus and at another position, which varies with the MHC molecule under inspection, within the peptide sequence[3,4]. Free peptides have an extremely short half-life in the cytoplasm; thus the generation of appropriate peptides, their protected transport into the endoplasmic reticulum (ER) and loading onto MHC class I heavy chain molecules require a sophisticated system (Fig. 1).

After their cotranslational insertion into the ER membrane, MHC class I heavy chain proteins associate with the transmembrane chaperone calnexin, which assists folding before it can bind to β_2-microglobulin (β_2-m). The labile dimeric complex binds to a second chaperone, the lumenal ER protein calreticulin, which allows further folding and the association of class I heavy chains with tapasin. Tapasin has only been recently discovered and – like the class I heavy chain, *tap* and some proteasome genes – is encoded in the MHC

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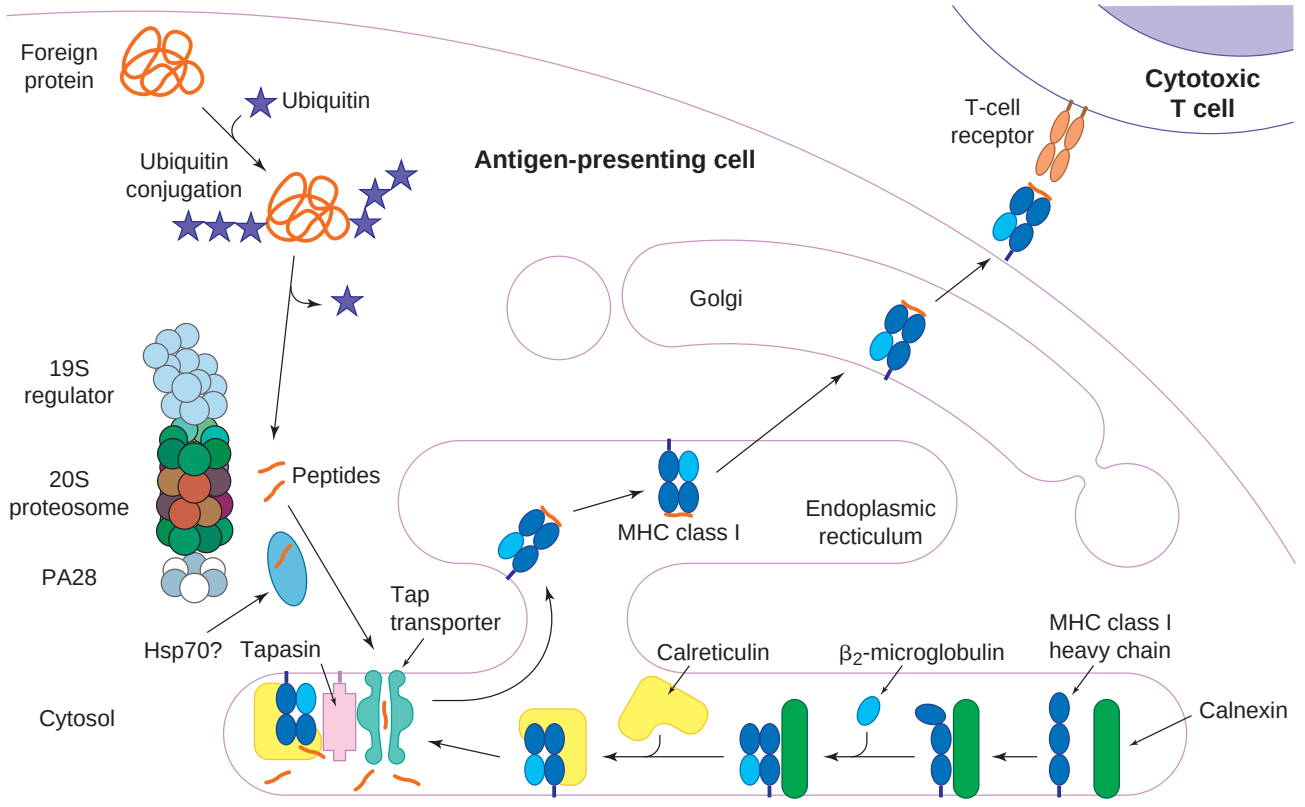

Figure 1. *Model of the major histocompatibility complex (MHC) class I pathway of antigen presentation. Nascent MHC class I heavy chain associates with chaperones calnexin – which allows partial folding and association with β_2-microglobulin (β_2-m) and calreticulin. Tapasin mediates binding of the calreticulin-bound class I heavy chain (β_2-m) dimer to the transporter associated with antigen presentation (TAP). Peptides are most likely generated from proteins targeted via ubiquitin conjugation to the proteasome complex. Proteasomes that simultaneously bind 19S regulator and PA28 might be involved in antigen processing. Peptides are conveyed by unidentified pathways (possibly Hsp70 mediated) to the TAP transporter, which allows translocation from the cytosol into the lumen of the endoplasmic reticulum (ER) lumen. Here they bind to the peptide-binding groove of class I molecules, which facilitates final folding, release from ER retention and migration to the cell surface. If the antigen receptor of cytotoxic T cells recognizes the appropriate MHC plus peptide (possibly derived from a virus protein), the T cell becomes activated to kill the infected target cell.*

gene locus. It mediates the binding of the class I heavy chain to the transporter associated with antigen presentation (TAP) and was found to coprecipitate with TAP, class I heavy chain, β_2-m and calreticulin[5–7]. The TAP-mediated transport of peptides from the cytoplasm into the ER is ATP-dependent and selective for peptides with a preferred length of 8–11 amino acids[8]. Appropriate peptide ligands are required for the final folding of class I heavy chains, which induces release from ER retention and facilitates migration to the cell surface. At the end of this pathway, peptide plus MHC can be recognized by the antigen receptor of CTLs, which can prevent the spread of a virus by eliminating those cells that present virus-derived peptides.

The bulk of class I peptide ligands and their precursors are produced in the cytosol by the proteasome[9–11]. This enzyme is well suited for this task as it does not degrade proteins to amino acids but releases peptide products ranging in length from four to 14 amino acids[12–14]. A role for the proteasome in antigen processing initially became evident from experiments with peptide aldehyde inhibitors of the proteasome[15], and was later confirmed with the more specific proteasome inhibitor lactacystin, a *Streptomyces* metabolite[16,17], which – as a result of a limitation in peptide supply – prevents the maturation and egress of classical class I heavy chains from the ER (Refs 18–21). How the peptides are transported from the

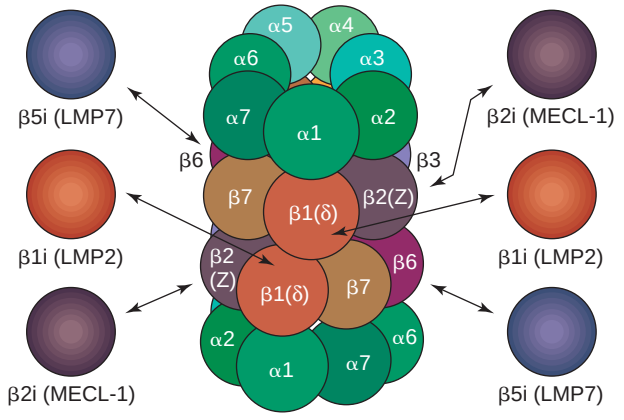

Figure 2. *Arrangement of subunits in the 20S proteasome and interferon-γ (IFN-γ) induced subunit exchanges. The IFN-γ inducible subunits MECL-1, LMP2 and LMP7 replace their respective constitutive homologues Z, δ and MB-1 in newly assembled 20S proteasomes. The order of subunits is deduced from the X-ray crystallographic analysis of* Saccharomyces cerevisiae *proteasome[23] and from crosslinking studies with human proteasomes[92]. The complex has a C2 axis between the β1 subunits of the inner two rings. The nomenclature is adapted from Ref. 23 and detailed in Table 1.*

Table 1. Nomenclature of 20S proteasome subunits

Traditional human	Traditional yeast	GDB[a]	Complex[b]
α-Type subunits			
Iota	PRS2	PSMA6	α1_sc
HC3	Y7	PSMA2	α2_sc
HC9	Y13	PSMA4	α3_sc
HC6 (XAPC7)	PRE6	PSMA7	α4_sc
Zeta	PUP2	PSMA5	α5_sc
HC2	PRE5	PSMA1	α6_sc
HC8	PRS1	PSMA3	α7_sc
β-Type subunits			
Delta (Y)	PRE3	PSMB6	β1_sc
LMP2		PSMB9	β1i_sc
Z (MC14)	PUP1	PSMB7	β2_sc
MECL -1		PSMB10	β2i_sc
HC10-II	PUP3	PSMB3	β3_sc
HC7-I	PRE1	PSMB2	β4_sc
MB1 (ϵ,X)	PRE2	PSMB5	β5_sc
LMP7		PSMB8	β5i_sc
HC5	PRS3	PSMB1	β6_sc
HN3 (β)	PRE4	PSMB4	β7_sc

[a]Nomenclature in Genome Database.

[b]Nomenclature according to the *Saccharomyces cerevisiae* 3D structure[23]: adjacent subunits are numbered sequentially within each ring from the subunit closest to the particle diad; α- and β-type subunits with equivalent numbers are adjacent.

proteasome to the ER is not well understood. As immunogenic peptides have been eluted from the heat shock protein Hsp70, it is clearly a possibility that chaperones transport the peptides while protecting them from degradation[22].

Structure of the proteasome and its active sites

Cell-cycle regulation, apoptosis, morphogenesis, differentiation, metabolic control and antigen presentation are key biological processes that are all controlled by the proteasome. Selective inhibition of catalytically active subunits or regulators of the proteasome with the aim of silencing one but not the other functions of the proteasome thus seems a formidable task. Understanding the complex structure of the 20S proteasome and its regulators is a prerequisite for drug design and has been greatly advanced by the recent X-ray crystallographic determination of the three-dimensional structure of *Saccharomyces cerevisiae* 20S proteasome at high resolution[23]. The 20S proteasome is a barrel-shaped core proteolytic complex consisting of four rings with seven subunits each (Fig. 2 and Table 1). Subunits of the inner two rings are of the β-type and bear the proteolytically active centres pointing towards the lumen of the barrel; the outer two rings are of the α-type and control access to the inner chambers of the proteasome and association with regulatory complexes. The β-subunits are synthesized as proforms, which are cleaved during assembly of the proteasome[24]. This process liberates a threonine residue at the N-termini of three mature β-subunits, and these contribute their γ-hydroxy groups as nucleophiles and their α-amino groups as proton donors/acceptors in the catalytic cycle. It is believed that the ϵ-amino group of a conserved Lys33 performs the residue proton donor/acceptor function of the initial processing step during assembly. Interestingly, a fourth mature β-type subunit (PSMB4 or HN3), bearing an N-terminal Thr1 but lacking Lys33, is not bound by the peptide aldehyde inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (LLnL). This is in contrast to Thr1 residues of the three putatively active subunits PSMB5 (MB-1, X), PSMB6 (δ, Y) and PSMB7 (MC14, Z), which form hemiacetals with LLnL in soaked crystals. Thus, it is only these latter three subunits that are likely to directly execute the cleavage of peptide bonds[23].

Traditionally, the activities of proteasomes were measured with fluorogenic oligopeptides and were classified, according to the nature of their P1 residue, as chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolytic

(PGPH), and it was assumed that these activities could be assigned to the unique catalytically active β-type subunits. In a careful mutagenesis study in *S. cerevisiae*, Heinemeyer and coworkers replaced the Thr1 residues of all three β-subunits bearing active sites with alanine, which should render them inactive. With this approach, a remarkably clear assignment of peptide-hydrolysing activity to β-subunits was possible: PSMB5 (Pre2) for chymotrypsin-like activity, PSMB6 (Pre3) for PGPH activity and PSMB7 (Pup1) for trypsin-like activity[25]. However, as residues other than P1 are known to determine substrate binding and proteasomal peptidolytic activity, the classification of proteasome activities according to P1 residues of fluorogenic peptides, which are shorter than most natural proteasome products, seems less than perfect and does not properly reflect the results obtained with polypeptide or protein substrates[14].

Targets for manipulating antigen processing?

Exchanges of 20S proteasome subunits

Vertebrate species that possess a cytotoxic immune response and MHC class I restricted antigen presentation also have an additional level of complexity in proteasomal antigen processing. Upon stimulation of cells with the antiviral cytokine interferon γ (IFN-γ), three additional proteasome subunits of the β-type are expressed that are homologous to the three putatively active β-subunits and that replace their constitutive homologues in newly formed proteasomes[10,11,26–30]. In mutant cell lines and knockout mice, deficiency of these inducible subunits leads to a slightly reduced cell-surface expression of MHC class I molecules and to a modest reduction in class I restricted presentation of certain antigens[31,32]. The incorporation of these inducible subunits changes the cleavage pattern of isolated proteasomes *in vitro*[14,33–36]. For one of these subunit exchanges we have a good molecular conception as to why this exchange occurs: replacing the subunit PSMB6 (δ) with the IFN-γ-inducible PSMB9 (LMP2) reduces the C-terminal cleavage of glutamic acid (PGPH activity) and enhances the C-terminal cleavage of hydrophobic amino acids. This makes sense for antigen processing because glutamic acid is found within MHC class I ligands but never at their C-terminus. By sequence alignment of these subunits with the homologous subunits of *S. cerevisiae*, it emerges that the pocket that binds the P1 residue of peptide substrates in PSMB6 accommodates negatively charged residues, whereas the P1 pocket of PSMB9 prefers hydrophobic residues.

However, the hope that structural information and sequence alignment would functionally elucidate the two other exchanges of PSMB8 (LMP7) for PSMB5 (MB1) and PSMB10 (MECL-1) for PSMB7 (Z, MC14) has been dashed because no dramatic change in the P1 pocket can be predicted. Moreover, results of *in vitro* digestions with isolated immunoproteasomes from different laboratories have been contradictory[10,14,33–39]. Simple assignment of tryptic activity to the PSMB7 (Pup1) subunit, as found for yeast mutants, does not appear to hold true for the murine PSMB7 subunit (MC14) and its IFN-γ-inducible homologue (MECL-1) (M. Groettrup, unpublished). Importantly, a proof-of-principle that silencing a single active site of the proteasome can modulate antigen presentation without eliminating essential housekeeping functions of the proteasome has recently been obtained. The constitutive mouse proteasome subunit PSMB6 (δ) was replaced in transfected cells by an overexpressed PSMB9 (LMP2) subunit bearing a site-directed Thr→Ala mutation at the N-terminus. The replacement affected MHC class I cell surface expression and the presentation of a mouse cytomegalovirus antigen while viability and proliferation of the transfected cells remained unchanged[40].

Certain human alleles of the IFN-γ-inducible subunit PSMB9 (LMP2) correlate with the severity of autoimmune diseases such as ankylosing spondylitis[41] and juvenile rheumatoid arthritis[42]. It would thus be valuable to have high-resolution structural information on human 'constitutive proteasomes' and 'immunoproteasomes' (containing IFN-γ-inducible subunits) for drug design and, potentially, to advance our understanding of the function of these subunit exchanges.

Exchanges of 20S proteasome regulators

Three regulatory complexes that bind to the 20S core proteolytic complex have been identified so far. The 19S regulator – also designated PA700 – binds to the α-endplates of the 20S proteasome to form the so-called '26S proteasome', a 2000 kDa complex which plays an essential role in the degradation of ubiquitin-conjugated substrate proteins. Six of the 17 different PA700 subunits are members of the triple-A family of ATPases, and it is believed that ATP hydrolysis provides the energy for unfolding native proteins in order to feed them into the 20S proteasome[43]. To what extent ubiquitin conjugation is a prerequisite for antigen processing is still a matter of debate[44–46].

A second regulator that also binds to the α-ends of the 20S proteasome cylinder is the '11S regulator' or 'PA28' (Refs 47,48). Electron microscopy and biochemical experiments have suggested that three copies each of an α- and a β-subunit form an alternating hexameric ring-like structure[49–51], although recombinant PA28α by itself forms a heptameric ring[52]. Both subunits of PA28 are inducible by IFN-γ (Ref. 53), and overexpression of PA28α greatly enhances the presentation of viral model antigens, suggesting a role for PA28 in

antigen processing[54]. The latter notion is further supported by the fact that PA28α/β is only found in species that have an MHC class I restricted antigen-presentation system. Interestingly, the potent immunosuppressive agents cyclosporin A, FK506 and rapamycin were recently found to downregulate the expression of PA28α and -β, which might influence antigen processing and add to the immunosuppressive effect of these compounds[55]. The presence of PA28 *in vitro* caused isolated 20S proteasomes to alter their cleavage behaviour: in the presence of PA28, MHC ligands were generated from polypeptide substrates much earlier in the course of a digestion compared with processing by 20S proteasomes alone, where longer intermediates accumulated before a second cleavage generated the MHC ligand[14,56]. It was hypothesized that PA28 might coordinate the action of two juxtaposed proteasome subunits to perform dual cleavages that should produce nonameric or octameric peptides. We propose an alternative hypothesis to explain the observed findings: PA28 might alter the conformation of the proteasome such that peptides longer than nine amino acids are retained more efficiently in the lumen of the proteasome to allow further cleavage events to occur. The latter hypothesis is supported by recent *in vitro* findings that show that the length of a peptide will determine whether it is further processed or released from the proteasome[57]. However, whether this is the function of PA28 *in vivo* and what the respective contributions of PA28α and PA28β are, remain to be determined.

A third regulator of the 20S proteasome is the 'Ki antigen' or 'PA28γ'. The PA28γ protein is homologous to PA28α and -β but it is not found in mixed complexes with PA28α/β. Instead, it forms homomeric complexes (most likely hexamers), which also bind to 20S proteasomes[58,59] but which by contrast to PA28α/β are exclusively found in the nucleus[60]. A homologue of PA28γ has been detected in the brown ear tick *Rhipicephalus appendiculatus* and the nematode *Caenorhabditis elegans*, which are species that do not possess an MHC class I restricted immune defence[61,62]. For this reason, and because PA28γ is not inducible by IFN-γ, it is unlikely to function in antigen processing. Intriguingly, the PA28γ protein is even rapidly degraded in IFN-γ-treated cells by an unknown mechanism[58], which might facilitate binding of PA28α/β to 20S proteasomes. Apparently, the steady-state equilibrium of the 20S proteasome and its regulators requires careful adjustment to achieve an optimal antiviral immune response.

It has been a puzzle how PA28α/β could render antigen processing more effective under the premise that PA28α/β–20S proteasome complexes are unable to digest native proteins *in vitro*. One way out of this dilemma would be if native proteins could be targeted via the ubiquitin pathway to the PA700 regulator binding to one side of the 20S proteasome while PA28 could induce efficient antigen processing by binding to the other side (Fig. 1). Convincing evidence for the proposed existence of a 20S proteasome bound by two different regulators[10] has recently been obtained by Hendil and coworkers, who succeeded in coimmunoprecipitating the 20S proteasome and PA28α/β with a PA700 reactive monoclonal antibody[63].

Novel and selective proteasome inhibitors

Lactacystin: a modulator of antigen presentation?

Lactacystin is a natural *Streptomyces* metabolite, which after having been extensively tested for three years has proved to be exclusively selective for mammalian 20S and 26S proteasomes over a wide range of concentrations[16,17,64]. As proteasome function is essential for survival, a proteasome inhibitor is expected to be a killer. However, lactacystin was not discovered as a poison but as an agent that induces cell differentiation – namely, the outgrowth of neurites and arrest in cell cycle of a mouse neuroblastoma cell line (Neuro2A) after treatment for 2–4 days at low concentrations (1.3 μM). Only at higher concentrations (10 μM) was lactacystin toxic for Neuro2A cells[65,66]. Moreover, depending on the model system and, more importantly, the concentration used[67], lactacystin was able either to induce[68] or to prevent[69,70] apoptosis.

By contrast to man-made inhibitors, lactacystin is not a modified peptide but contains other structural elements from which it is named: a γ-lactam ring, a cysteine and a hydroxyisobutyl group[16]. Lactacystin itself does not inhibit the proteasome. Rather, it loses *N*-acetyl-L-cysteine in aqueous solutions to form clastolactacystin β-lactone, which in turn is able to permeate cell membranes and to inhibit the proteasome (Fig. 3a)[71,72]. The β-lactone ring is attacked by the β-hydroxy group of N-terminal threonine residues of β-type proteasome subunits to form a stable ester between the inhibitor and the enzyme. Interestingly, it was predominantly the PSMB5 (MB-1, X) proteasome subunit that was labelled when extracts from bovine tissues were incubated with 10 μM radioactive lactacystin. The PSMB7 (MC14, Z) subunit was labelled much less efficiently and the PSMB6 (δ, Y) subunit not at all[17]. Similar results were obtained by X-ray analysis of lactacystin-soaked proteasome crystals where only PSMB5 (PRE2) bound by lactacystin could be detected[23]. These data seem consistent with the tentative assignment of proteasome activities to unique subunits in yeast because the association constant for lactacystin inhibition of the chymotrypsin-like activity was 20- and 50-fold higher than for the trypsin-like and PGPH activities, respectively. It appears that lactacystin at low concentrations (1–3 μM) is a selective inhibitor of the chymotrypsin-like activity, and only at a

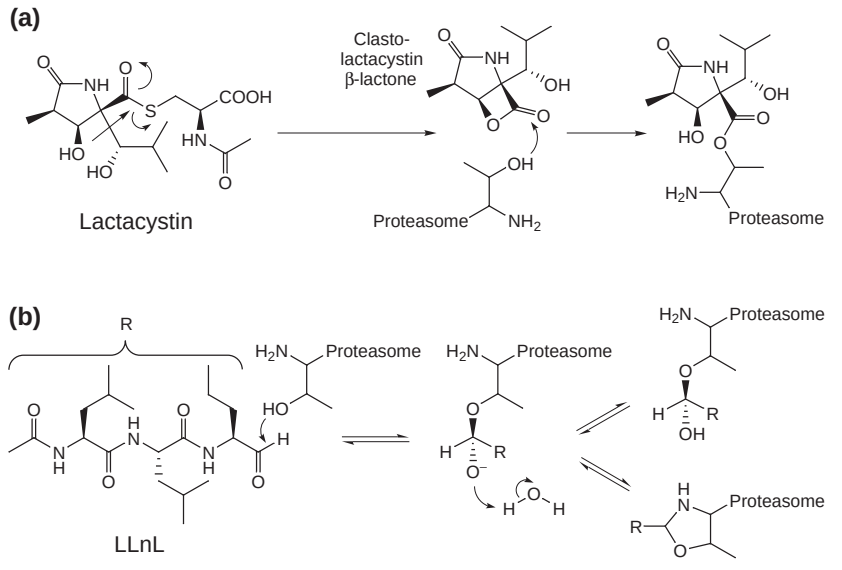

Figure 3. *Mechanism of covalent proteasome inhibition by (a) lactacystin and (b)* N*-acetyl-leucyl-leucyl-norleucinal (LLnL).*

concentration of 100 μM were all active proteasome β-type subunits including the IFN-γ-inducible ones labelled by radioactive lactacystin[19].

The effect of lactacystin on MHC class I restricted antigen presentation has been tested for several influenza virus T-cell epitopes derived from nucleoprotein, haemagglutinin, NS1 protein and matrix protein. For all these peptide epitopes, antigen presentation was markedly inhibited by 10 μM lactacystin and completely abrogated when treated with 100 μM of the inhibitor[18]. In the lymphocytic choriomeningitis virus (LCMV) system, the presentation of four epitopes from the viral glycoprotein and nucleoprotein was eliminated by treatment with 10 μM lactacystin without the production of virus proteins being affected (Ref. 21; K. Schwarz and M. Groettrup, unpublished). Even the generation of an epitope derived from the ER leader of the LCMV glycoprotein was strictly proteasome dependent[21]. For the presentation of an ovalbumin epitope, a concentration of 2.4 μM lactacystin or 1.2 μM clastolactacystin β-lactone was sufficient to block antigen presentation completely[19]. Taken together, it appears that most of the T-cell epitopes tested so far, including those derived from ER-targeted viral glycoproteins, are sensitive to lactacystin, albeit at different ranges of concentration. Very recently, two epitopes from influenza virus in human and murine presentation systems have been reported for which lactacystin enhanced rather than prevented class I restricted antigen presentation[73,74]. It has been suggested that the epitopes are generated by another proteolytic system (which has not been identified) and that the proteasome destroys the epitopes by performing cleavages within the relevant nonameric peptide sequence[75]. However, enhancement of antigen presentation by lactacystin could also be explained by the selective inhibition of active sites of the proteasome, resulting in the production of different peptide fragments. Consistent with this hypothesis, the half-life of influenza nucleoprotein was not significantly altered in cells treated with 10 μM lactacystin, but antigen presentation was enhanced[73]. The interpretation of these results is complicated by the finding that proteasome inhibition leads to enhanced production of cell stress proteins such as Hsp70 and grp94, which are potentially involved in antigen processing. Thus, further *in vitro* and *in vivo* experiments will be required to establish whether lactacystin at low concentrations can act as a selective modulator (rather than a plain inhibitor) of antigen presentation.

Peptide-derived inhibitors

Virtually all man-made inhibitors designed to target the proteasome are chemically modified oligopeptides. Specificity for the proteasome or even for single proteasome subunits was obtained either by varying the type of amino acids used at the respective P1, P2, P3 or P4 position or by replacing the C-terminus with different functional groups with the aim of covalently and irreversibly binding to the active sites. A principle emerging from several studies is that inhibitors containing three or four amino acids achieve better inhibition than inhibitors with only two amino acids[76,77]. X-ray analysis of proteasome crystals with a bound inhibitor containing six amino acids revealed that the residues in P1–P3 position could be positionally defined, whereas the N-terminal residues were more mobile[78].

The first generation of proteasome inhibitors were peptide aldehydes that form covalent hemiacetals with threonine of the active site (Fig. 3b). As the aldehyde group also reacts with active sites of cysteine proteases, the proteasome specificity of these reagents was quite variable, a fact nicely documented by the original name and function of the prototype of these compounds, *N*-acetyl-leucyl-leucyl-norleucinal (LLnL): calpain inhibitor I. For the chymotrypsin-like activity of the proteasome (K_i = 5.7 μM), LLnL is about 10 and 40 times more potent than for the trypsin-like and PGPH activities, respectively (Table 2)[79]. As a calpain inhibitor, however, it is 50-fold more potent. As LLnL penetrates cell

membranes easily, it was initially used to study the function of the proteasome, but these experiments required careful titration of the inhibitor to ensure that the observed effects were not due to the inhibition of cathepsins or calpain[15]. More recently developed peptide aldehyde inhibitors designed with modified amino acids achieved K_i values in the submicromolar range both for the chymotrypsin-like activity of the proteasome and for calpain[15,76,80,81].

Much more potent and selective inhibitors have been obtained by replacing the C-terminal aldehyde group by α,β-epoxyketones[82], vinyl sulphone[83], glyoxal[84,85] or boronic acid[84,86] (Fig. 4 and Table 2). Boronic acid compounds, for example, exploit the advantage that sulphur and boron bind only very weakly and by this means a 200,000-fold higher selectivity for the proteasomal chymotryptic activity (K_i = 0.03 nM) over the thiol protease cathepsin B was achieved.

Tetrapeptide vinyl sulphones have been synthesized that inhibit the chymotryptic activity >1000-fold more potently than the proteasomal tryptic or PGPH activity[83]. Using such an inhibitor (**3**, Fig. 4) in a radiolabelled form, Bogyo and coworkers showed that the proteasome subunits PSMB5 (MB-1, X), PSMB8 (LMP7) and PSMB9 (LMP2) were labelled most intensively, suggesting that they are the subunits contributing to the chymotrypsin-like activity of mammalian proteasomes[77]. In the same study, an inhibitor was designed that exclusively tagged the PSMB9 (LMP2) subunit, demonstrating that proteasome subunit-specific inhibition is feasible. Further interesting implications of this study were that the P4 residue could be important in targeting a peptide vinyl sulphone inhibitor to a particular proteasome subunit and that the binding site for the P4 position is sensitive to chiral geometry as exchange of a D-tyrosine for an L-tyrosine drastically reduced the inhibition. Moreover, competition experiments with cold inhibitors suggested that the subunits PSMB7 (MC14, Z) and PSMB10 (MECL-1) were at least in part responsible for the trypsin-like activity of the proteasome.

A recent library screening pursued a different approach: rather than aiming at covalent modification of active site threonines, competitive binding to proteasomal substrate binding sites was the goal. A series of peptides with a 5-methoxy-1-indanone-3-acetic acid group at the N-terminus was identified that proved to be selective for the chymotrypsin-like activity of the 20S proteasome (IC_{50} = 0.2 μM), while other proteasomal proteolytic activities or calpain were not inhibited[87]. For most of these recently developed proteasome inhibitors, their biological effects in general and their impact on antigen presentation in particular have not yet been reported. This will be interesting and perhaps even surprising.

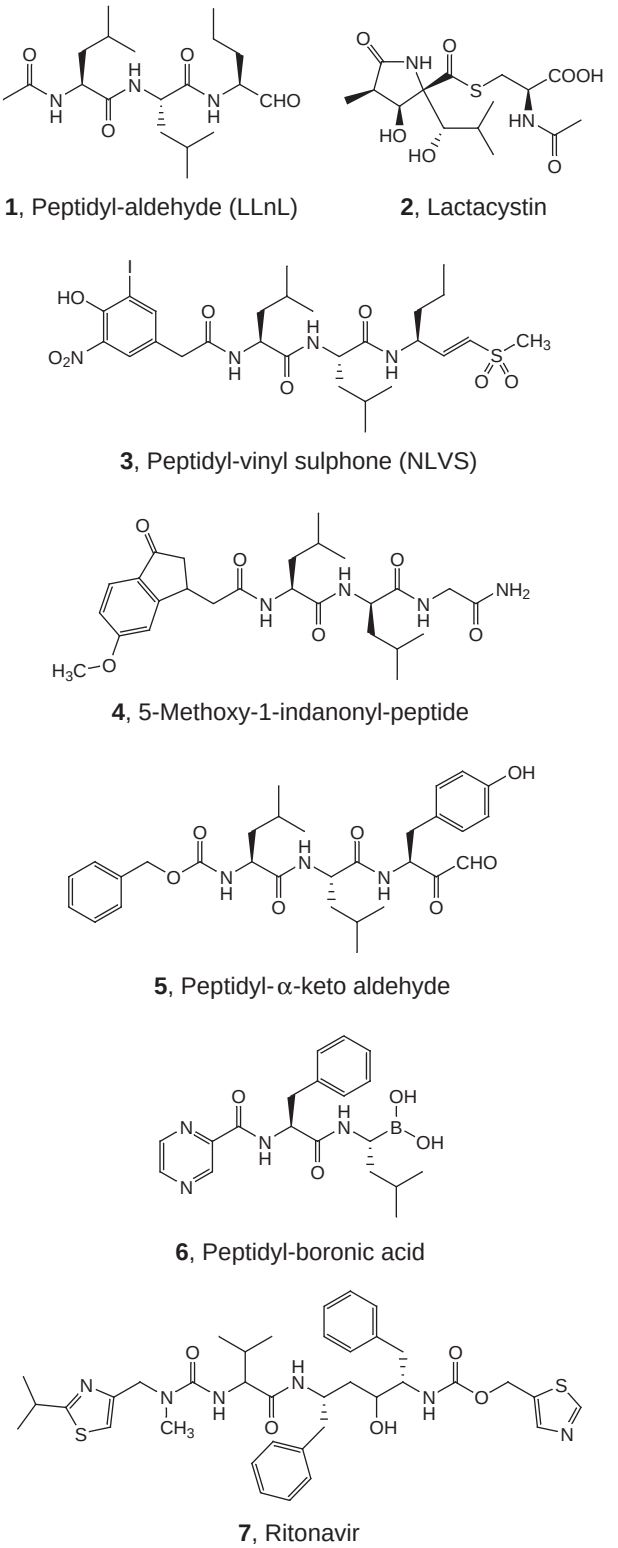

Figure 4. *Structure of selected proteasome inhibitors. Some characteristics of these inhibitors are listed in Table 2.*

Table 2. Comparison of potency and selectivity of proteasome inhibitors[a]

Compound	ChLP	TrLP	PgLP	Other proteases	Refs
1	K_i = 5.7 μM	K_i = 50 μM	K_i = 205 μM	Calpain I, K_i = 0.12 μM	79,93
2	IC_{50} = 0.8 μM	IC_{50} = 10 μM	IC_{50} >100 μM	Not inhibited	17
3	IC_{50} = 0.3 μM			Cathepsin S labelled	83
4	IC_{50} = 0.2 μM			Calpain I not inhibited	94
5	K_i = 3.1 nM				85
6	K_i = 0.62 nM			Cathepsin G, K_i = 630 nM	86
7	IC_{50} = 3.0 μM	Activated	IC_{50} = 50 μM	HIV-1 protease, K_i <1.0 nM	91

[a] K_i or IC_{50} refer to inhibition of the proteasomal chymotrypsin-like activity (ChLP) – hydrolysis of Suc-LLVY-MCA (100 μM); trypsin-like activity (TrLP) – hydrolysis of Bz-VGR-MCA (400 μM); and PGPH activity (PgLP) – hydrolysis of (Z)-LLE-βNA (100 μM). Blank spaces indicate that data were not available.

Selective inhibition of the proteasome and antigen presentation

Inhibitors of the protease encoded by HIV-1 allowed a great leap forward in the clinical treatment of AIDS (Ref. 88). A recent study evaluating clinical data of patients treated with HIV-1 protease inhibitors and nucleoside analogues for over one year yielded an extraordinary finding: in ~40% of patients a steady increase of $CD4^+$ helper T cells was observed in spite of the fact that virus titres in the blood did not decline (probably because of viral drug resistance)[89]. It has been suggested that the decline of $CD4^+$ T cells in AIDS patients is a result of the elimination of HIV-1-infected $CD4^+$ cells by HIV-1-specific CTLs. Consistent with this concept, preservation of $CD4^+$ cells in viraemic patients might result from inhibition of CTL-mediated killing by an HIV-1 protease inhibitor. This hypothesis, initially proposed by our collaborators André and Lotteau, was tested and confirmed in the mouse model by Klenerman and Zinkernagel[90]. Generation of the cytotoxic immune response against LCMV was markedly reduced in infected mice treated with the HIV-1 protease inhibitor ritonavir (Fig. 4)[91]. The typical *in vivo* expansion of LCMV-specific $CD8^+$ CTLs was not observed in ritonavir-treated mice, and viral titres were elevated in the course of the immune response. Subsequent experiments with LCMV-infected cells showed that ritonavir inhibited the MHC class I restricted presentation of LCMV glycoprotein and nucleoprotein epitopes. In search of a molecular target for ritonavir, we tested whether the proteasome would be affected. Surprisingly, ritonavir inhibited a chymotrypsin-like activity of isolated murine or human 20S proteasomes (measured with the substrate Suc-LLVY-MCA) with an IC_{50} of 3 μM, which is similar to the proteasome inhibitors, lactacystin and LLnL. By contrast to general proteasome inhibitors, ritonavir moderately enhanced the trypsin-like activity, whereas it barely affected the proteasomal PGPH activity. Treatment of cultured cells with higher concentrations of ritonavir (50 μM) led to cell cycle arrest, to the intracellular accumulation of ubiquitin conjugates and to inhibition of lipopolysaccharide-induced degradation of the proteasome substrate IκB (Ref. 90). Thus, ritonavir inhibits antigen presentation at a concentration where vital housekeeping functions of the proteasome, such as cell division, are not yet blocked.

Although it is not yet possible to prove that proteasome inhibition is the molecular reason why MHC class I restricted antigen presentation is dampened by ritonavir, our data strongly suggest that ritonavir may be a prototype of a drug that, by selective inhibition of the proteasome, modifies antigen processing. Compounds of this sort might be applied as specific immunosuppressive agents in autoimmune disease and transplantation medicine in the future. For the development and search for selective proteasome inhibitors this is only the beginning.

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